Phytoremediation of a highly creosote-contaminated soil by means of *Salix viminalis*

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ABSTRACT

The aims of this study were to investigate whether *Salix viminalis* could grow in a highly creosote contaminated soil and, if so, whether the introduction of the plant enhanced the dissipation of polycyclic aromatic hydrocarbons (PAHs). The purpose was also to examine whether inoculation of two different bacterial strains further enhanced the decrease of the contaminant. The experiment was conducted in a greenhouse during ten weeks. The soil was collected at a former wood impregnation site belonging to the Swedish railway administration, in Krylbo, Sweden. The experiment comprised two different levels of PAH contaminated soil, the creosote soil used as such or diluted with an arable soil (1:1). The main PAHs studied in this experiment were phenanthrene, anthracene, fluoranthene, pyrene and benzo[a]pyrene. The soil samples were extracted with toluene and analysed by GC-MS. Microbial analyses were made to measure the number of extracted and cultivable bacteria (CFU/g soil) and dominating bacteria were tested for gram, fluorescence and oxidase reactions. The plants were infected by some bacterial disease which probably was an inherent infection of the cuttings and the number of "healthy" plants was reduced by 2/3 in both the creosote soil and the diluted soil. The initial PAH concentrations were very high, particularly for fluoranthene and pyrene. Some PAH degradation occurred in pots without plants, but the dissipation of all studied PAHs was enhanced in the presence of *Salix viminalis* and also the reduction of more recalcitrant compounds such as benzo[a]pyrene was impressive. In the presence of plants the PAH compounds were reduced in the creosote soil by 67 %, 79%, 77% and 43 % for anthracene, fluoranthene, pyrene and benzo[a]pyrene respectively compared to the initial values and by 61 %, 66%, 79% and 30% in the mixed soil. For all studied PAHs the concentrations in the rhizosphere soil were found to be greater than in the bulk soil and this could probably be explained mostly by the movement of compounds from the surrounding soil into the rhizosphere, but partly also by an increased solubility. The results indicate that the introduction of the inocula was not succesful, but due to the inadequate number of pots with "healthy" plants no conclusions could be drawn. The microbial analyses showed a significantly higher number of cultivable bacteria per gram of soil in treatments with plants both compared to the initial soil and the treatments without plants.

SAMMANFATTNING

Syftet med examensarbetet var att undersöka *Salix viminalis* förmåga att växa i en kraftigt kreosotkontaminerad jord och huruvida växten bidrog till en ökad nedbrytning av polyaromatiska kolväten (PAHer). Avsikten var också att studera hur nedbrytningen påverkades av tillsats av två bakteriestammar som visat sig intressanta vad gäller nedbrytning av PAHer. Den kreosotkontaminerde jorden som användes i försöket kommer från ett område, Krylbo, där SJ tidigare bedrivit impregnering av slipers. Jorden innehåller höga halter av åldrade PAHer, med initiala värden överstigande 1000 ppm för t ex fluoranten och pyren. Den kontaminerade jorden användes som sådan samt späddes (1:1) med en okontaminerad jord insamlad strax utanför impregneringsområdet. Försöket pågick i växthus under tio veckor och provtagning skedde efter fyra, åtta och tio veckor i led utan plantor och efter tio veckor i behandlingarna med planta. De PAHer som i huvudsak studerades närmare under försöket var antracen, fluoranten, pyren samt benzo[a]pyren. Kompletterande mikrobiella analyser genomfördes med avsikt att beräkna bakteriepopulationer (viable count) samt isolera och gruppera de vanligast förekommande bakterigrupperna genom oxidas-, gram-, fluorescenstest. Två tredjedelar av plantorna påverkades så pass kraftigt av en bakterieinfektion att dessa fick uteslutas ur den slutliga bearbetningen av analysresultaten. Studien visade att en viss nedbrytning av fluoranten och pyren skett efter tio veckor i krukor utan växt, både i blandjorden och i den rena kreosotjorden. Resultaten visar en ökad nedbrytning av alla de studerade PAHerna i närvaro av *Salix viminalis* där koncentrationerna av antracen, fluoranten och pyren reducerades med 67, 79 respektive 77 % i den rena kreosotjorden och med 61, 66 respektive 79 % i blandjorden jämfört med intitialjorden. Benzo[a]pyren minskade med 43 % i kreosotjorden och med 30 % i blandjorden, vilket är ett lovande resultat då benzo[a] pyren tillhör de mer svårnedbrytbara PAHerna. De reducerade koncentrationerna kan vara en följd av en ökad mikrobiell aktivitet i jorden och därmed en ökad mibrobiell nedbrytning. Detta resonemang stärks av resultaten från viable count som visar på ett ökat antal bakterier i behandling med växt. Andra eventuella orsaker till den ökade nedbrytningen kan vara växtens produktion av biosurfaktanter, vilka ökar lösligheten av PAHerna och därmed tillgängligheten för mikrobiell nedbrytning. PAH koncentrationerna för alla PAHer i studien konstaterades öka i rhizosfärjorden jämfört med jorden i hela krukan, vilket troligen främst kan förklaras av en ökad rörelse mot rotzonen genom vattenupptag, men också en ökad löslighet av PAHerna kan vara en bidragande orsak. Det gick inte att se någon effekt på nedbrytningen till följd av bakterieinokuleringen, men på grund av det begränsade antal friska plantor och en stor variation mellan replikaten kan ingen slutsats dras utav detta. Undersökningen visar på *Salix viminalis* förmåga att både överleva i den kraftigt kontaminerade jorden och dess förmåga att bidra till PAH-nedbrytningen. Ytterligare försök är dock nödvändiga för att kartlägga de bakomliggande mekanismerna.

CONTENTS

INTRODUCTION

Over the past century approximately 40 000 contaminated sites have been identified in Sweden according to the Swedish Environmental Protection Agency (Naturvårdsverket, 2003). The biological and ecological consequences of chemical pollution from industrial activity are serious and are amplified throughout the ecological food web. Because of the importance and the great extent of this problem, significant research and practical effort are directed toward developing improved methods for remediating environmental contamination.

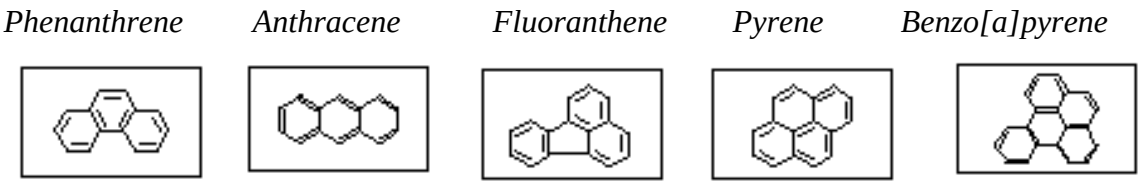
Creosote, which is a common preservative used in wood treating processes, consists to 85 % of different polycyclic aromatic hydrocarbons (KemI, 2005). Because of their toxicity, and their mutagenic and carcinogenic properties polycyclic aromatic hydrocarbons are classified as ubiquitous contaminants in the environment of highest concern (Aprill & Sims, 1990; Wilson & Jones, 1993; Kanaly & Harayama, 2000).

Biological remediation methods utilize microorganisms to accomplish the degradation. The ultimate goal of any degradation process is complete mineralization of the organic contaminants, where carbon dioxide, water and other inorganic compounds are formed. Several studies have shown enhanced degradation of polycyclic aromatic hydrocarbons in the presence of plants (Aprill & Sims, 1990; Reilley *et al*., 1996; Alexander, 1999; Siciliano *et al.*, 2003; Newman & Reynolds, 2004) and these results look promising.

BACKGROUND

Polycyclic aromatic hydrocarbon

Polycyclic aromatic hydrocarbons, from now on termed PAHs, are a group of non polar hydrophobic chemicals that are formed during the incomplete combustion of coal, oil, gas wood, house hold or other organic substances, such as tobacco and charbroiled meat (Wilson & Jones, 1993). They are made up of two or more fused benzene rings in a linear, angular and cluster arrangement. Nitrogen, sulfur and oxygen atoms may substitute for carbon in the benzene rings to form heterocyclic aromatic compounds (Mueller *et al.*, 1996). The structures of the main PAHs in this study are shown in *Figure 1*.



Figur 1. Structures of the PAHs in this study adapted from Jinno Laboratory (2005).

Sources of PAHs in the environment

PAHs are naturally, but during the industrialization of the world the emissions of PAHs have shown a considerable increase and become a problem of great environmental concern (Wilson & Jones, 1993). The contaminated areas are generally not more than a few hectares, but the PAH concentrations are mostly high and often in association with other types of hydrocarbons, xenobiotic chemicals and trace metals (Mueller *et al.*, 1996). High concentrations of PAHs in soil are for example a result of spillage and dumping of creosote, which is a common preservative, used in wood treating processes. Creosote consists to 85 % of close to 100 different PAHs (KemI, 2005).

PAH characteristics and degradation

PAHs have a low water solubility and a high solubility in organic solvents, and the concentration in the soil water phase is thus quite low. But PAHs represent a long-term source of water pollution because they will continue to enter the water phase to replace what is transported away from the site, degraded, or removed by some remediation technology (Alexander, 1999). The solubility in water of the PAHs in this study is given in Table 1 below.

Table 1 Number of aromatic rings, water solubility and molecular weight of PAHs discussed in this study.

Compound (PAH)	*Number of rings*	*Solubility (mg/l)*[1]	*Molecular weight*[2]
Phenanthrene	3	1.300	178
Anthracene	3	0.070	178
Fluoranthene	4	0.260	202
Pyrene	4	0.140	202
Benzo[a]pyrene	5	0.003	252

[1]Mueller *et al.*, 1996 [2]Lundstedt, 2003

The biodegradability of PAHs is dependent on the chemical structure of its various components. In general, the lighter, more soluble PAHs are more biodegradable than the heavier, less soluble members of the group. For example, phenanthrene, which is considered more easily biodegradable than pyrene, has a water solubility of 1.3 mg/l compared to a solubility of 0.14 mg/l for pyrene. Similarly, pyrene is more biodegradable than benzo[a]pyrene, which has a solubility of less than 0.003 mg/l (Lundstedt, 2003). PAHs with four or five rings are usually highly persistent (Alexander, 1999). A compound's resistance to biodegradation increases with increasing molecular weight. Two-ring compounds such as naphthalene are broken down faster than four-ring compounds such as pyrene (Mueller *et al.*, 1996).

Also the steric arrangement of the aromatic rings affects their environmental stability and hence their natural distribution. Simpler chemical structures are easier to degrade. For example, compounds with branched benzene rings, like phenanthrene, are less stable than the linearly arranged anthracene molecules and do not in general survive in nature unless they become trapped or bound to certain organic or inorganic compounds (Mueller *et al.*, 1996). This generalization about the relationship between structure and biodegradability is applicable in aerobic environments, but experiments under anaerobic conditions are still poorly understood (Alexander, 1999).

Some chemicals may be toxic to the microbes. In some cases, compounds that are readily biodegradable at low concentrations may be toxic to microorganisms at high concentration levels (United-tech., 2005). Microbial communities have been shown to be far less diverse in soils containing large amounts of PAHs and in the paper of Cheung & Kinkle (2001) the authors bring up the results of other reports indicating that environmental stresses, including contamination, not only reduce the biodiversity (defined both as the number and variety of organisms) of the original community but may also selectively enrich specific microorganism populations that are more adapted to the new environment.

The potential of biodegradation may also be affected by the composition of other pollutants, the mixture of PAHs, and PAHs mixed with other types of compounds. If a multitude of compounds are present even easily metabolized compounds can be more recalcitrant (Alexander, 1999). Many studies have been performed under controlled conditions in the laboratory with pure bacterial strains and spiked soils, but these conditions hardly reflect the real conditions at contaminated soil sites.

Bioavailability

Bacterial cells are excluded from soil pores smaller then 0.2-0.8 μm and predation, by for example protozoa and nematodes, is believed to reduce the bacterial biomass in pores larger then 2μm (Hartel, 1999). Consequently a large fraction of PAH degrading bacteria in soil will be physically separated from the PAH sources due to restrictions of the microbial mobility. PAH degrading populations in soil are probably not growing for most of the time, due to carbon- and energy starvation, and only maintenance growth will occur, i.e. bacterial growth replaces decaying cells (Johnsen *et al.*, 2005). Burrowing soil animals such as nematodes, springtails, mites and earthworms contribute to an increased bioavability of the PAHs through their mixing of the soil but they may also take up the PAHs through the body surface. PAH uptake also occurs through feeding, since PAHs are adsorbed to the soil organic matter (Johnsen *et al.*, 2005).

The soil variables of most importance for the bioavailibility of organic pollutants are the clay content and the content of organic material (Lundstedt, 2003). Clay adsorbs mainly the polar organic compounds and metal ions through cation exchange or other adsorption processes (McBride, 1994). PAHs sorb to a greater extent to organic matter, like “humic acids”, than to the clay constituents in the soil, due to the hydrophobic and non-polar characteristics of the PAHs (Alexander, 1999), which make these compounds relatively unavailable for microbial degradation (Lundstedt, 2003). PAHs also bind to hydrophobic groups on dissolved organic matter (DOM), which represents all organic material dissolved in soil water, or they adsorb to hydrophobic particles that have colloidal properties (Alexander, 1999). Colloids are particles which do not sedimenting in soil water (size 1nm-10μm) and the colloids bind the PAHs through van der Waal forces. These mechanisms affect the translocation and transport of the PAHs in the soil and the PAHs may be transported for considerable distances throughout the soil (Marwin *et al.*, 2004).

Organic compounds that persist in soil often undergo a time dependent decline in bioavailability and the biodegradation rate becomes slow during the aging process. This phenomenon occurs both in the field and under laboratory conditions (Alexander, 1999). Initially, a certain PAH compound disappears as a result of biodegradation, but after it has resided in the soil for some time, little or no biodegradation occurs. Because most polluted soils were contaminated many years ago aging is of particular interest. The rate and extent in the aging of a compound differ between different soils. In soils with a high content of organic matter there is a declining aging rate (Alexander, 1999). During aging PAH molecules may be entrapped within the solid phase of the organic matter and become resistant to desorption and thus also inaccessible to microbial degradation (Alexander, 2000). It has also been suggested that the bioavailability of entrapped molecules may be reduced due to precipitation of minerals which blocks small pores (Farrell *et al.*, 1999). The aging is also affected by the initial degradation rate of the compound. With a low initial degradation rate the compound will be less available with time (Alexander, 1999).

PAH metabolism

Degradation of PAHs in the ambient environment can occur through biological, chemical and photochemical processes. Biological degradation appears to be the main process responsible for the PAH removal in soil. Three metabolic processes are identified as having the most significant roles in microbial degradation of PAHs: aerobic- and anaerobic catabolism and cometabolism. Some PAH degrading microorganisms, primarily bacteria, are capable of using several PAHs as their sole carbon and energy source (Wilson & Jones, 1993). In cometabolic reactions, transformation of a specific contaminant occurs indirectly, i.e., as the result of the metabolism of another substance, which otherwise could not be metabolized by the bacteria themselves (Hickey, 1999). The presence or absence of oxygen is a major factor for determining the direction and rate of the degradation. Depending on which microorganisms are present and their relative abundance and activity, different metabolic processes will occur and different products will be formed and thereafter accumulate and persist at a bioremediation site (Alexander, 1999). Aerobic biodegradation is currently the most common form of bioremediation practised in soils contaminated with PAHs (Wilson & Jones, 1993) and so far most research has been carried out under aerobic conditions.

The first step in the bacterial degradation of PAHs is the activation of dioxygenase, which incorporates two oxygen atoms into the aromatic ring of a PAH molecule resulting in the formation of *cis*-dihydrodiol which is then further oxidised to dihydroxy intermediates. These intermediate products then undergo cleavage and form TCA-intermediates (Wilson & Jones, 1993). Once a contaminant has been enzymatically transformed to a less complex compound, it can often be metabilized further through various pathways (Skipper, 1999).

Degradation of PAHs may also be carried out by fungi. Bacteria and fungi have different metabolic pathways and unlike bacterial degradation, fungal degradation does not require that the contaminant is incorporated by the fungi before degradation can occur. Fungi

produce monooxygenases instead of dioxygenases and one oxygen atom is incorporated into the aromatic ring to form arene oxides. This step is followed by the enzymatic addition of water to form phenols and *trans*-dihydrodiols (Wilson & Jones, 1993). General pathways for microbial degradation of PAHs are shown in Figure 2.

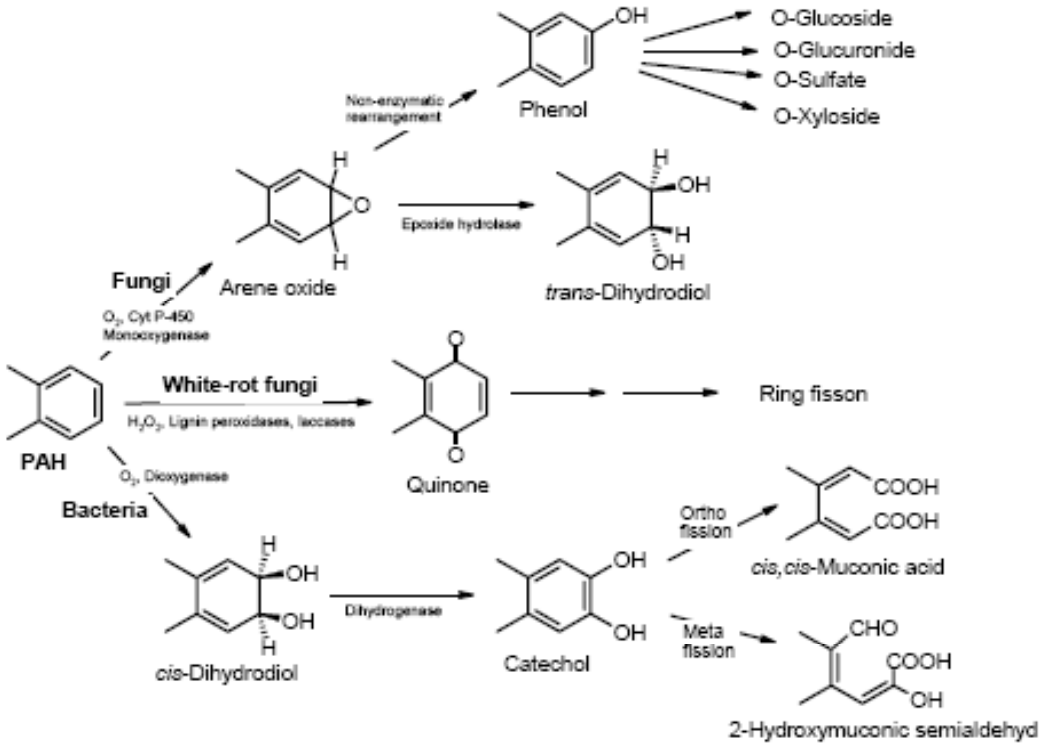


Figure 2. General pathways for microbial degradation of PAHs adapted from Cerniglia, (1992).

There have been many reports of the degradation pathways of low molecular weight PAHs, such as naphthalene and phenanthrene, but during the latest decades biodegradation of PAHs composed of more than three aromatic rings has also been studied (Kanaly & Harayama, 2000; Samantha *et al.*, 2002).

It has been found that several coexisting bacterial strains degrade pollutants more efficiently than a single strain due to the presence of bacteria which use various intermediates degradation products more efficiently (Kupier *et al.*, 2004).

Degradation metabolites

The ultimate result of PAH degradation is carbon dioxide and water as end products. Both in biological and chemical degradation processes various metabolites might be formed on the way to complete mineralization (Lundstedth, 2003). Metabolites are formed both under highly controlled laboratory conditions favourable for degradation, and in the field, but it is not very easy to keep track of the metabolites and trace their origins in field. The metabolites may include temporary intermediates but also

compounds that are more resistant to further degradation (Lundstedth, 2003). These metabolites generally possess a higher polarity than the original compounds and are thus more soluble and bioavailable to the microbial community (Johnsen *et al.*, 2005) but this also results in increased mobilization of the compound and thus a risk for further spreading and leakage (Johnsen *et al.*, 2005). The toxic and mutagenic properties of the intermediates might not change, or in the worst case they will increase, compared to the toxicity and mutagenecity of the parent PAHs (Alexander, 1999). Some PAHs will be toxic and mutagenic only after degradation activation.
The potential accumulation of metabolites of PAHs is often ignored during soil remediation programmes (Lundstedt, 2003) and many of these products are probably temporary intermediates, but due to their possible toxicity, known or unknown, and the fact that they are constantly formed at contaminated sites it is of great importance to take these compounds into account.

Reference values and guidelines
In order to predict the environmental and potential health risks of a contaminated soil site, it is necessary to quantify the contamination levels. Assessments of contamination levels are based on the extent to which these levels result in an environmental risk or are hazardous to human health, and are also used for defining reference values. The latter values represent the concentration of substances, which would be found at a site if it were not contaminated and are supposed to reflect natural concentrations. Urban areas usually have higher reference values than in rural areas. Reference values reflect current environmental conditions, and have nothing to do with levels that are regarded as desirable or levels that have negative effects (Naturvårdverket, 2002). The Swedish Environmental Protection Agency has set up guidelines for contaminated sites, which are based on what is currently known about PAHs, to protect people and the environment from possible health and other negative effects. These guidelines were published in 1996, and in 2003 they were complemented with new restrictions regarding the use of creosote in order to have the same restrictions throughout the European Union (Naturvårdsverket, 2003). The general reference values for carcinogenic PAHs are 0.3 mg/kg and are set to 20 mg/kg dw for other PAHs (Naturvårdsverket, 1996). Guidelines for compounds of interest in this study are listed in Table 2. It is of importance to point out that the guidelines are not supposed to represent a level up to which it is allowed to contaminate. It should be noted that the contamination levels of PAHs in the investigated soil far exceeded those guidelines (see results).

Table 2. Guideline values for PAHs compounds (Naturvårdsverket, 1996).

	Phenanthrene	*Fluoranthene*	*Pyrene*	*Benzo[a]pyrene*
Concentration (mg/kg dw)	0.5	1	0.6	0.4
	Total PAH	*Carcinogenic PAHs*		*Other PAHs*
	5	2.5		2.7

Methods for remediation

Because of their complex molecular structure, PAHs are some of the most difficult compounds to degrade biologically (Naturvårdsverket, 2002). If sites contaminated with PAHs are just abandoned, which often has been the case in the past, degradation of the contaminant may occur, but often at a slow rate. The distribution of PAHs in soils is very heterogeneous and distribution, movement and degradation rate are dependent on the local environmental conditions (Mueller *et al.*, 1996). Many creosote contaminated sites formerly accommodated industrial enterprises, and as a result of steadily growing cities these sites are nowadays often located within urban areas. The unpredictable and in many cases hazardous fate of the contaminants and the risk of exposure to humans and animals have resulted in requirements for remediation of the contaminated sites.

Conventional techniques for cleaning up contaminated soil may include chemical and/or physical methods like chemical fixation, soil washing, *in-situ* thermal treatment, incineration of excavated soil, or disposal in landfills (Wilson & Jones, 1993). These techniques are generally effective, but often at the cost of a permanent negative effect on surrounding ecosystems. The cleaning may require the use of costly chemicals or equipment. Alternative techniques are being developed to allow for a less destructive and more complete contaminant removal that would allow a better ecosystem recovery. In many cases, a combining of different remediation methods will give the best result.

Bioremediation

Bioremediation is defined as ”a strategy or a process that uses microorganisms, plants, or enzymes produced by plants or microorganisms to detoxify contaminants in the soil and other environments” (Skipper, 1999). The degradation processes may be enhanced by changing chemical or physical conditions in the soil, such as pH, moisture, aeration and nutrient content. Enhancement could also be achieved either by adding specifically adapted microorganisms, introduce suitable plants or favor the biological degradation in other ways .

The bioremediation strategies shown below are cited from Skipper (1999) and include:

- *Passive* or *intrinsic bioremediation*, which is the ”natural” bioremediation of a contaminated site carried out by the indigenous microorganisms,

- *Biostimulation* is the stimulation of indigenous microorganisms through the addition of nutrients or an extra carbon source,

- *Bioventing* means that gases such as oxygen or methane are added to the soil to stimulate different types of microbial activity,

- *Bioaugmenting*, is the inoculation of either a single bacterial strain or a consortium of several species at the contaminated site,

- *Landfarming* is the incorporation and mixing of the contaminant with an uncontaminated soil,

- *Composting* is the process of using aerobic, thermophilic microorganisms in constructed piles of soil, mixed with organic material, to degrade the contaminants;

- *Phytoremediation* will be discussed below.

Bioremediation is highly dependent on site conditions and soil properties. Whether a compound is biodegradable or not is not the same as saying that it will be biodegraded.The following criteria must be fulfilled:

- Suitable microorganisms must be present at the site,

- Limiting inorganic nutrients must be present or added,

- The compound must be in a bioavailable form as it may be largely unavailable if it is sorbed or sequestered in other ways.

- No toxic substances affecting microbial growth and activity are present at the site,

- The concentration of the contaminant must be above the threshold level for the microbial populations using it as a C and energy source and

- Suitable chemical and physical environmental conditions including pH, organic matter content, soil type, water content and climate regime must be fulfilled. (Alexander, 1999).

Phytoremediation

Phytoremediation is the use of plants to remediate contaminants in the environment. Several studies have shown the beneficial influence of plants, but the mechanisms involved are still poorly understood (Aprill & Sims, 1990; Reilley *et al.*, 1996; Qui *et al.*, 1997; Alexander, 2000; Siciliano *et al.*, 2003; Newman & Reynolds, 2004). A microbe might be very sensitive to one or two site variables but plants are generally more able to cope with a broad range of soil conditions. Phytoremediation is based on biological processes and involves the capability of plants and associated microbial populations to capture, accumulate, or break down contaminants, or to reduce contaminant mobility. The potential economical and environmental benefit of phytoremediation is frequently mentioned when recommending this technique (Aprill & Sims, 1990; Reilley *et al*., 1996; Glick, 2003; Kupier *et al.*, 2003; Vervaeke *et al.*, 2003; Newman & Reynolds, 2004).

A number of different plant species have been found to stimulate the degradation of organic compounds including many common wild grasses, maize, wheat, soybean, peas and beans (Glick, 2003). Several tree species, such as willow and poplar species may also contribute to the degradation of organic contaminants (Kupier *et al.*, 2004). These plants mostly have extensive and fibrous roots and form extended rhizospheres (Glick, 2003).

In an investigation made by Aprill & Sims (1990) a greater remediation efficiency was found in soil planted with several prairie grass species compared to unvegetated controls. Similarly, PAH degradation was favoured in another study, by the presence of several grass species and alfalfa. Anthracene and pyrene declined from 100 ppm to less then 14 ppm in four weeks and degradation in the vegetated soil was enhanced by 30 to 44 % compared to unplanted soil (Reilley *et al.*, 1996). The degradation rate of pyrene was found to be enhanced by more than 30 % in the presence of several different plant species, among them both field crops, horticultural crops and tree seedlings (Liste & Alexander, 2000a).

The main difficulty with phytoremediation is to find plant species that is able to degrade or accumulate a specific compound. It is also of importance that the plant should affect the contaminant without affecting the surrounding ecosystem to any great extent.

The plant must of course be able to grow in the presence of the pollutant and must tolerate local conditions at the site including for example toxic levels of trace metals, the ambient climate conditions, pH and water and oxygen supply. It is also of importance to consider the size, activity and species composition of the rhizosphere microbial community as well as the soil volume exploited by the rhizosphere (Alexander, 1999).

PAHs are usually unevenly distributed in heterogeneous media such as soil and this will affect the degradation conditions. Phytoremediation is only useful in situations where the plant roots can reach the contaminant. Therefore this technique is not a universal solution.

The common mechanisms and phenomena exploited for phytoremediation include the following ones:

Phytoextraction
Pollutants are taken up from the soil and concentrated in aboveground plant tissues. This is primarily applicable to soils contaminated with trace metals (Glick, 2003). According to Gao & Zhu (2004) it has been suggested that PAHs and other lipophilic organic pollutants, are strongly associated to the soil organic fraction, and not expected to be taken up by plants and translocated. This assumption is confirmed by the earlier results of Aprill & Sims (1990) and Reilley *et al.* (1996) who concluded that the plant translocation of PAHs is negligible. It has been shown, however, that some plants that grow on PAH contaminated soils may contain PAHs in their tissues (Fismes *et al.*, 2002). Gao and Zhu (2004) themselves found a positive correlation between root lipid contents and the concentration of phenanthrene and pyrene in root tissues of different plant species. A study on PAH degradation carried out with vegetables grown on industrially contaminated soils showed that PAH concentrations in plants tended to increase with PAH concentrations in soil (Fismes *et al.*, 2002). In another study it was found that the lipids in roots were the major reservoir for water insoluble contaminants. It was also shown that the most lipophilic organic compounds were located in the epidermis of the roots and that higher lipophilicity resulted in higher root concentrations (Chiou *et al.*, 2001). These results were confirmed in the study of Gao and Zhu (2004) where a larger root uptake could be found for pyrene compared to phenanthrene and this was suggested

to be explained by the higher lipophilicy of pyrene. The root uptake is mainly through adsorption processes rather than by root uptake in the strict sense (root absorption). Unlike high molecular weight PAHs, which are strongly adsorbed on root epidermis cells, low molecular weight PAHs are able to migrate to shoots (Fismes *et al.*, 2002).

Phytotransformation

The results of phytoextraction discussed above indicate an uptake and translocation of the PAHs but the future fate of the PAHs in the plant are not fully known. However, it is known that plants can degrade contaminants. This occurs either through PAH uptake and detoxification by enzyme systems within plant cells, or through exoenzymes that are excreted into the soil and act outside the plant (United-tech., 2005). Plants may produce peroxidases and biosurfactants, such as saponin (Hong *et al.*, 2002), and these substances are known to be involved in the direct degradation of PAHs (Yoshitomi & Shann, 2001). Degradation of anthracene has been observed within the cortex cells of both maize and wheat roots (Wild *et al.*, 2005), but whether this is true for other PAHs is not documented so far.

Rhizosphere biodegradation

The microbial activity in the rhizosphere is intense and commonly found to be 10- to 50 times higher, than in the root-free soil (bulk soil). In general the proportion of gram negative bacteria increases in the rhizosphere (Paul & Clark, 1989). Microbial activity has been considered as the most influential and significant cause of PAH removal (Daane *et al.*, 2001). The microbes in the rhizosphere consist of a diverse community, with different types of metabolism. These microbes are well adapted to the environmental conditions and PAH degrading bacteria have been isolated from the soil rhizosphere (Binet *et al.*, 2000; Maya & Firestone, 2000). Miya and Firestone (2000) observed a greater percentage of PAH degrading bacteria in rhizosphere soil than in bulk soil and suggested that the rhizosphere selected for PAH degraders. Previous studies showed that the number of bacteria using aged creosote as a sole carbon source was higher in the rhizosphere than in the bulk soil (Sisciliano *et al.*, 2003).

Enhanced degradation of PAHs in rhizosphere soil has been reported by Liste & Alexander (2000b), Daane *et al.* (2001) and Yoshitomi & Shann (2001). The disappearance of organic pollutants in the root zone occurs due to stimulation and modification of the microbial community of the rhizosphere (Aprill & Sims, 1990; Reilley *et al.* 1996; Gao & Zhu, 2004; Kupier *et al.*, 2004). It is thought that several processes contribute to these observations. Organic compounds in the root exudates may act as carbon and nitrogen sources, and support the growth and metabolic activity of the microbes capable to degrade organic pollutants (Aprill & Sims, 1990). Many plants secrete compounds that are structurally similar to important pollutants, which favour bacteria that can utilize both these exudates and the contaminant. The release of plant root exudates has been observed to enhance the removal of the recalcitrant benzo[a]pyrene (Rentz *et al.*, 2005). Plants may also influence PAH degradation through their introduction of root litter, which will increase the carbon availability in rhizosphere (Miya & Firestone, 2001). Plants may also affect the microbial community through

changes in pH, oxygen supply or water content and there will be changes in on soil structure due to root development and penetration of the soil matrix.

Studies showing an increased concentration of the contaminant in the rhizosphere have also been reported (Liste & Alexander, 2000b). The mechanisms involved remain poorly understood, but these results are not contradictory to those discussed above. The hypothesis is that an enhanced microbial degradation occurs in parallel with a PAH flow towards the roots. This PAH flow has not been demonstrated but has been discussed by Qiu *et al.* (1997) and Liste & Alexander (2000b). The solubility of the PAHs is enhanced in the rhizosphere (Qui *et al.* 1997) and this may also explain a higher PAH concentration in the rhizosphere. Roots are known to produce enzymes such as peroxidases (Liste & Alexander, 2000b) and these enzymes are known to increase the water solubility of PAHs through inducing an oxidation of the aromatic rings (Wilson & Jones, 1993).

Phytostabilization

A variety of mechanisms can reduce contaminant migration. In some cases, reduction of mobility is a sufficient treatment. Insoluble contaminants are usually less toxic (Wilson & Jones, 1993). Plants can provide fixation of contaminants through absorption and accumulation by roots, adsorption onto roots, or precipitation within the root zone of plants and the use of plants and plant roots to prevent contaminant migration via wind and water erosion, leaching, and soil dispersion (Gas Technology Institute, 2005). Phytostabilization will not directly degrade the organic contaminants, but the microbial activity associated with the plant roots may enhance the degradation rate. Phytostabilization also refers to establishing a plant cover on the surface of the contaminated soil or sediment, with the intention to reduce the exposure of the soil to humans or animals (Naturvårdsverket, 2003).

Surfactants

To increase the solubility and enhance PAH degradation mobilizing agents such as surfactants can be used (Mueller *et al.*, 1996). A surfactant molecule consists of a hydrophobic and a hydrophilic part and is soluble in water at low concentrations, but at higher concentration it forms micelles towards the water phase with their hydrophilic part, (Alexander, 1999). It has been shown that a number of PAHs, for example phenanthrene, anthracene and pyrene will be desorbed from soil particles when surfactants are present (Mueller *et al.*, 1996). Boonchan *et al.* (1998) suggested based on earlier results that surfactant addition may enhance the degradation of not only the more soluble PAHs but also the more recalcitrant five- and seven- ring compounds such as benzo[a]pyrene and coronene.

High concentrations are needed to desorb PAHs with a low water solubility, but even low concentrations of surfactants seem to enhance the mineralization of the compounds even if the compounds are not desorbed (Alexander, 1999). The impact of surfactants may not always improve the degradation rate of PAHs and in some cases there may even be a negative impact (Mueller *et al.*, 1996). In the paper by Wilson and Johnson (1993) the authors bring up the problems associated with the surfactant use, i.e. the toxicity and degradability of the surfactant. They also discuss the research to overcome these

problems. For example, high concentrations of surfactants may inhibit microbial activity, or microbes will preferentially degrade the added surfactants instead of the contaminants.

Plants and microorganisms produce biosurfactants, which may increase the bioavailibility of sparingly available substrates as PAHs. For example, PAH degrading *Pseudomonas* strains produce surfactants which increase the solubility of the contaminant (Johnsen *et al.*, 2005). In an investigation by Willumsen & Karlsson (1997) it was found that several PAH degrading bacteria, isolated from PAH contaminated soils, release biosurfactants under experimental conditions. Plant-produced biosurfactants, such as saponins, were found to enhance the removal of trace metals in an investigation carried out by Hong *et al.* (2002), but plant derived biosurfactants are also suggested to be involved in the degradation of PAHs (Ressler *et al.*, 1999). Biosurfactants may also influence the bacterial uptake of PAHs. According to Johnsen *et al.* (2005) the uptake of biosurfactant-solubilized molecules was found to be faster than the uptake of truly dissolved molecules.

Inoculation

If the indigenous microorganisms do not act sufficiently rapidly to prevent the spreading of a pollutant or the microorganisms acting on certain pollutants are absent from particular sites inoculation may be an alternative. For example Juhasz *et al.* (2000) showed a degradation of a single high molecular weight PAH with up to 98 % of pyrene and 26 % of benzo[a] pyrene in a liquid medium after inoculation with a *Stenotrophomonas maltophilia* strain. By using a mixture of PAHs they found concurrent degradation, although at a lower extent, of high and low molecular weight PAHs using the same bacterial strain.

A *Sphingomonas sp.* strain inoculated in a spiked soil showed degradation of anthracene in a previously sterilized soil, but the degradation rates were only enhanced compared to those of the indigenous bacteria community at neutral soil pH (Kästner *et al.*, 1998).

Mycobacterium spp. are commonly isolated from PAH contaminated soils and increased degradation of several PAHs has been found after inoculation of a *Mycobacterium* strain, but the degradation rate differed between different PAH levels of the contaminated soil. It was also found that the effect on the PAH degradation of this *Mycobacterium* strain was greater after initial sterilization of the soils, which suggests that competition with indigenous bacteria affected the degradation activity of the strain (Cheung & Kinkle, 2001).

The advantage of any inoculation depends on the microorganism survival and performance in the new environment. This may include limitations in growth due to competition with indigenous microorganisms, lack of substrate and suboptimal experimental conditions or site conditions (Wilson & Jones, 1993).

Most studies are done under laboratory conditions, but a successful outcome of inoculation in the field was described by Mueller *et al.*, 1996. The authors reported results of an *in situ* trial carried out by Rosenberg *et al.*(1992). The inoculated bacteria were selected for their capacity of degrading the contaminant but also for their ability to degrade a unique source of organic nitrogen added to the soil, i e. a modified urea-

formaldehyde polymer,which was not readily utilized by the indigenous microbial community (described in Mueller *et al.*, 1996).

The inoculum may contain a microbial mixture or a single strain. The most commonly reported bacterial genera associated with PAH degradation include: *Bacillus,* cyanobacteria, *Flavobacterium, Mycobacterium, Pseudomonas* and *Rhodococcus* (Wilson & Jones, 1993).

The size of the inoculum is considered as sufficient at 10^7 to 10^8 cells /g to establish a measurable degradation activity, but this amount rests on the assumption that the survival automatically leads to degradation activation. Kästner *et al* (1998) showed that a considerable amount of bacteria survived in soil without developing their degradation capacity.

One of the greatest limitations to *in situ* inoculations involves the problem of getting the bacteria to reach the contaminant and/or the contaminant to reach the bacteria. Physical mixing of the soil, such as tillage, may be required (Mueller *et al.*, 1996).

AIM

The aims of this study were: (i) to examine whether willow, *Salix viminalis*, could grow in a highly contaminated aged creosote soil and if so (ii) to examine whether the degradation of polycyclic aromatic hydrocarbons (PAHs) is enhanced by the presence of willow and (iii) to examine whether inoculations with two different bacterial strains further enhance the dissipation of the contaminants.

MATERIALS and METHODS

The sampling site at Krylbo

The contaminated soil was collected from a former wood impregnation site at Krylbo (60º 07’ 14’’ N, 16º 13’ 35’’ E). A sampling spot where low concentrations of arsenic were previously detected was chosen to avoid phytotoxicy problem caused by arsenic. The Swedish railway administration, ‘Banverket’, has used the site from 1911 to 1986, for impregnation of sleepers using creosote in oil. For years, liquid process wastes containing creosote were applied to the soil (Sandström, 2005 pers.com). A map of the Krylbo site is shown in appendix.

Degradation trials with the creosote contaminated soil were conducted by Sweco VBB Viak AB, from July 1993 to October 1994 to explore the possibility of microbial degradation. These trials resulted in a reduced soil toxicity and a decrease in the majority of the PAHs. In the evaluation Sweco VBB Viak AB concluded that there seem to be promising possibilities for microbial degradation in situ.

Plant material
Willow (*Salix viminalis*, clone 78 183) cuttings were obtained from the Department of Short Rotation Forestry, SLU. This clone was chosen since it has been used with promising results in earlier studies at our Department (Hultgren, 2004; Mastera, 2004).

The cuttings were placed in tap water three days before planting to initiate sprouting.

A pilot trial was conducted during ten weeks to investigate whether the willow clone could grow in such a highly creosote contaminated soil. Using a unpolluted control soil the creosote soil was diluted at the mass ratios 1:10 and 1:1 and used as such and two cuttings per dilution rate were planted. Also, two plants were planted in the control soil. All plants survived but the above ground biomass (data not shown) and chlorophyll content (Figure 3) decreased with increased creosote content and visual signs of contamination effecs, like deformed leaves and side shoots, were observed on plants growing in the undiluted creosote soil.

Soil preparation
The experiment comprised two different levels of PAH contaminated soil: 100% contaminated soil and a mixture (50% contaminated soil) with a control soil. The mixture was based on the dry weights of the soils. The control soil was an arable soil from a site at Ulleråker, Uppsala, which was not contaminated with PAHs or other pollutants. The soils were sieved through a 2-mm sieve and water content, water holding capacity and pH were determined. The creosote soil had an initial pH of 6.2 and the control soil had a pH of 7.7. The pH values did not differ/change much throughout the experimental period in either the control soil, the mixed soil or the creosote soil (data not shown). The water holding capacity (WHC) was obtained by the following procedure. Tubes with a nylon net in the bottom were filled with soil and put in a water bath for 24 hours and then drained for a few hours. The samples were weighed, dried at 105°C and weighed again. The WHC was 48 % of the soil dry weight in the creosote soil, 39% in the mixed soil and 29 % in the control soil.

Experimental description
The soils and soil mixtures described above were set up with untreated controls and inoculated with two bacterial species, both with and without willow. All treatments were repeated four times. For the treatments with plants 1 liter pots were used and 700 g dw of soil was transferred to the pots. Treatments without willows were prepared in 250 ml pots, containing 300 g dw of soil, except for one of the replicates for which the larger pot size was used (see Table 3).

Table 3. Description of treatments and number of pots used in the greenhouse experiment. Bact A: *Rhodococcus wratislaviensis*, Bact B: J04 isolate from Mastera (see below) (2004), [1];250 ml pots.

Treatment[*]	*Sampling week 4*	*Sampling week 8*	*Sampling week 12*
Creosote soil	4[1]	4[1]	3[1] + 1
Creosote soil + Bact A	4[1]	4[1]	3[1] + 1
Creosote soil + Bact B	4[1]	4[1]	3[1] + 1
Mixed soil	4[1]	4[1]	3[1] + 1
Mixed soil + Bact A	4[1]	4[1]	3[1] + 1
Mixed soil + Bact B	4[1]	4[1]	3[1] + 1
Creosote soil + plant	-	-	4
Creosote soil + plant + Bact A	-	-	4
Creosote soil + plant + Bact B	-	-	4
Mixed soil + plant	-	-	4
Mixed soil + plant + Bact A	-	-	4
Mixed soil + plant+ Bact B	-	-	4
Control soil + plant	-	-	4
Control soil + plant + Bact A	-	-	4
Control soil + plant + Bact B	-	-	4

[*]The control soil contained no PAHs and was thus not included as a soil treatment.

Inocula

Strain J04 was isolated from a spiked PAH soil (Mastera, 2004) and was obtained from the Department of Microbiology, SLU. J04 is a gram negative, oxidase positive and non fluorescent bacterium, suggested to be a member of *Sphingomonas* (Mastera, 2004). It will be termed J04 in this report.The *Rhodococcus* sp. DSM 44126 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, 2005) and was further identified as *Rhodococcus wratislaviensis* by 16s rDNA gene sequenseing (Pizzul, 2005 pers.com.).

Both strains were cultivated on agar plates (tryptone soya broth 10%) (MERCK) medium per liter and incubated at 25°C. After three days two loops of bacteria were transferred into Erlen Meyer flasks containing 100 ml of tryptone soya broth (30%) medium. Three replicates were made for each bacterial strain. The Erlen Meyer flasks where incubated at 25°C for three days, constantly shaking at 180 rpm. The bacterial cultures were harvested in the log phase by centrifuging at 5000 rpm for 10 min and resuspended in phosphate buffer containing (per l) 23.99 g NaH_2PO_4 and 15.59 g Na_2HPO_4. The growth was monitored by measuring optical density at a wavelength of 600nm (OD_{600}), using a Novaspec® II spectrophotometer (Pharmacia), and adjusted with the buffer to a value of 0.6, i.e. a bacterial concentration about 10^7 microbes /ml. The bacteria were inoculated to the soil at a final concentration of 2×10^8 cells/g of soil and the water content of the soil was adjusted to 60% of the WHC.

Plant maintenance

The pots were regularly watered each second day, with some exeptions, to maintain a water content of approximately 60 % of WHC throughout the experiment. A complete

nutrient supply (Blomstra) was made to pots with plants three weeks after planting, when nutrient stress was observed, and thereafter continuously when watering at a dilution of 2.5 ml/l. The added nutrients in pots with plants were assumed to be consumed by the plants and due to this assumption no corresponding addition was made to pots without plants.

Sampling

Samplings were initially planned at week 4, 8 and 12, but were made at week 4, 8 and 10, in all treatments without plants. For the treatment with plants sampling was only made at the end of the trial (see Table 3). When sampling, the soil was mixed in a plastic bag and approximately 5 g was transferred into a tube equipped with screw lid. The tubes were frozen at –20°C until extraction and analysis.

Final sampling

The pots with plants were treated in the following order: The leaves were cut off from the stem and weighed; The stem was cut off close to the soil; Roots were removed from the soil and gently shaken to remove loosely attached soil; The roots were then rinsed from soil adhering to the roots; This latter soil fraction was regarded as rhizosphere soil and was used for PAH analyses and for microbial determination, isolation and identification; The roots were then washed with tap water; Both leaves and roots were air-dried for one week and thereafter kept in paper bags at room temperature for later analyses; The bulk soil was treated in the same way as the rhizosphere soil .

Extraction

The soil samples were extracted with 20 ml toluene and 10 ml 0.05 M pyrophosphate and shaken for 16 hours at room temperature (modified from Karstensen, 1996). The samples were centrifuged at 2000 ppm for 15 min. Two ml of the supernatant was cleaned through an alumina column (International Sorbent Technology. Isolate® AL-N. Part No. 714-0020-B) and collected in a cryo tube, diluted at the ratio 1:10 (v/v) in toluene and kept at –20°C until analysis.

Analytical methods

PAH analysis

The PAH concentrations were measured with a Hewlett Packard model 6890 gas chromatograph. PAH injections were separated on a HP-5MS 19091S-433 (ChromTech) capillary column (30 m, by 0.25 mm [inside diameter] with a 0.25 μm coating phase). The oven temperature was programmed at 80°C for 4 min and increased with 7°C/ min until a final temperatur of 310°C was reached. The retention time data were complemented with mass spectral (MS) data obtained from an a Hewlett Packard model 5973 Mass Selective Detector.

The compounds were identified and quantified by comparing their peak areas in samples and reference standards using mixed analytical standards prepared in toluene, at concentrations covering the range 0.25 – 25 ppm. The mixed standards contained pure phenanthrene (MERCK), anthracene (MERCK), antraquinon, pyrene (ALDRICH) and benzo(a) pyrene (SIGMA) in exact concentrations. Naphthalene was added to the

standard vials in a quite high, but not exact, concentration to prevent the column from being saturated by the substances in the samples and thereby become contaminated. The peak areas and their retention times were also compared with an ISO certificated standard, retaining nine PAHs. The standard was purchased from ISO 9001 certified Reference Materials.

Isolation and identification of soil bacteria

Soil bacteria were extracted from the soil by mixing 1 g of soil with 100 ml of phosphate buffer containing (per l) 23,99 g NaH_2PO_4 and 15,59 g Na_2HPO_4 and 1 ml 10 % Calgon (KEBO) in a Braun house-hold mixer for 2 min, rest 5 min and run 1min. The soil particles were allowed to sediment for 5 seconds and 2 ml of the supernatant was transferred to 18 ml of phosphate buffer for further dilutions and plated on agar media (tryptone soya broth 30%). The number of colony forming units (CFU) was determined after three days of incubation at 25°C. After counting, the most common, or in other ways interesting, culturable bacterial strains were isolated.

The isolates were cultivated, at 25°C for 24 hours, on agar plates with Kings B medium (King *et al.*, 1954), containing (per l) 1.5 g K_2HPO_4, 1.5 g $MgSO_4$, 20 g proteose, 10 g glycerol and 15 g agar, for gram- and fluorescence tests and on Nutrient Agar medium (OXOID) for oxidase tests. Gram reaction was tested through mixing a drop of 3% KOH and a loop of bacterial colonies on a microscope slide with a toothpick. Gram negative properties of the bacterial strain are obtained if a thread is formed between the toothpick and the microscope slide. Fluorescent properties were tested on a CROMATO-VUE® (ultra-violet products, inc.) instrument at wavelength 365 nm and the oxidase tests were carried out with the Bactident® Oxidase test (MERCK). On the basis of these reactions the isolated bacteria strains were grouped (Hultgren, 2004). Enrichment and isolation of PAH-degrading bacteria was also carried out by transferring 1 ml of the supernatant to Erlen Meyer flasks containing 50 ml of sterile tap water with several PAHs, as the sole carbon and energy source, in a concentration of approximately 75 ppm. After 10 days bacteria were grown on agar plates (tryptone soya broth 30%) and the dominant strains were isolated and checked for gram-, fluorescence- and oxidase reactions (see above).

Statistical analyses

All data are means of the analysed replicates. Reported levels of variance are mainly based on 95% confidence limits (2×SE). Statistically significant differences ($P<0.05$) were determined using a two-tailed Students t-test.

RESULTS and DISCUSSION

Plant establishment and development

The cuttings in the main experiment were infected by some bacterial disease which was probably an inherent infection of the cuttings. Most of the plants showed no symptoms during the first few weeks after planting. Then plants in the mixed soil seemed to grow less well. This was assumed to be a consequence of the PAH content of the soil together with the interference from the control soil. However, after nine weeks, 2/3 of the plants in

the creosote soil suddenly started to wilt. The experiment therefore had to be terminated after ten weeks, which was two weeks earlier then planned. Dead or dying foliage remained attached to the plants. The roots of the infected plants had a brown colour. Symptom development and bacterial activity differed between the soil treatments. Symptoms observed on the plants in the creosote soil were initial stress, wilting of foliage and death of the entire plant within a few days after initial symptoms were observed. Plants in the mixed soil did not wilt suddenly, but had a constantly lower shoot biomass production rate compared to both plants in the control soil and in the creosote soil, and also in this case 2/3 of the plants were classified as affected or dying at the time of harvest. It should be mentioned that all of the plants which did survive in the creosote soil had developed white water roots at the soil surface, which indicates that the plants suffered from water stress. These water roots were at first assumed to be an effect of a higher water content of the soil due to less uptake of water by the plants, but this alternative could be excluded after measuring the soil water content. The water stress was likely to be a result of insufficient water transport through the vascular tissues due to the bacterial infection. In the mixed soil such water roots were not observed on any of the plants.

Both shoot and root biomass were measured as planned (further information presented below). All plants in both the creosote soil and the mixed soil had brown chloroses and cracks along the bark, but the symptoms were more obvious for the affected plants. These symptoms were also observed on a few plants in the control soil, but to a much lower extent. No differences were visually observed between the bacterial treatments within the same soil treatment. Due to the bacterial infection and its consequences it was necessary to decide whether the results of pots with affected plants should be excluded or not and to investigate if the total biomass production, degree of infection and level of contaminants in the soil were in any way related to the disappearance of PAHs in the respective soil.

In the creosote soil the plants could easily be divided into two groups, since eight plants out of 12 were clearly dying and had a much lower biomass production than the others. The four remaining plants were classified as healthy. Healthy plants does not mean that they were without bacterial infection and showed no phytotoxic effects, but they clearly differed from the more affected ones. The plants in the mixed soil were more difficult to separate into healthy and affected plants. Even though total biomass and visual observations corresponded well compared to the infection degree no clear transition between healthy and affected plants was obvious, but 2/3 of the plants in the mixed soil were classified as affected.

Since the majority of the plants were classified as affected, it was investigated whether the different bacterial treatments were significantly different or if it was possible to combine these treatments for overall conclusions. When comparing the bacterial treatments it was found that the variability was high, but the same pattern was found in pots without plants and no conclusions could be drawn due to the inadequate number of pots and errors originating from the analytical procedure (see below). Due to this, the plant effect of PAH degradation will from now on be related to the four healthy plants in the creosote soil and the same number of healthy plants in the mixed soil. These plants

originate from different bacterial treatments and the bacterial treatment will therefore not be taken into account when discussing the results of the plant effect (unless especially mentioned in the text).

Bacterial strains were isolated from the cuttings to establish a possible bacterial infection. Cuttings from the creosote soil, with clear symptoms, were compared with cuttings from the control soil where the symptoms were not obvious. The latter cuttings were used as controls to establish the occurrence and the differences in infection dergee, but no quantifications were made. After isolation, identification of the bacterial pathogens was made by simple gram-, fluorescence- and oxidize tests. The results are presented in the appendix. A correlation between visual phytotoxicity symptoms, like chlorosis and discolouration of plant leaves, and the degree of the bacterial infection was observed for the plants in the contaminated soil. Less infected plants corresponded also to better development of both above- and below ground tissues (data not shown).

A positive correlation between the total plant biomass (shoot + root biomass) and the disappearance of PAHs in the soil was found for anthracene, fluoranthene and pyrene in the mixed soil. The disappearance of benzo[a]pyrene was also enhanced by plant introduction but no relation to biomass development was found in this case. In the creosote soil a similar tendency was found but the correlation was only significant for pyrene.

Shoot and root biomass

The surviving plants in the mixed soil and particularly those in the creosote soil showed the capability of willow to grow in this highly contaminated soil. The biomass data are shown in Table 4. In this experiment a relationship was apparent between biomass production and degradation. However, Liste & Alexander (2000a) did not find any relationship between either total biomass, shoot or root biomass, or the ability of the plant to enhance the degradation rate of pyrene for oat, lupine, rape, dill, pepper, radish or white-, red- and black pine.

In general both the shoot- and root biomass were low and since this was also valid for plants in the control soil it was assumed to be mainly a result of the bacterial infection. The above ground biomass production for *Salix viminalis*, both in the control soil of the pilot trail (data not shown) and based on results reported by Hultgren (2004), was found to be approximately two times the biomass production in the main experiment. The effect of the degree of contamination was also established by the increased number of highly affected plants in the contaminated soils and the fact that the highest number of dying plants was found in the creosote soil, but also by the retarded plant development and the increased visual phytotoxicity symptoms in the contaminated soils. These conclusions are also confirmed by comparing the plant biomass in this trial with the earlier pilot experiment, conducted during the summer 2004, with equal amounts of soil and cutting material. Over all the plants grew less well in the main experiment both in the creosote soil and the control soil which confirms the effect of the bacterial infection, but the pilot trial also showed a clear relation between plant development and contamination degree (data not shown).

Table 4. Shoot- and root biomass (fw) (h); healthy plants, (a); affected plants. Values in parentheses are 95% confidence limits.

	Shoot (g)	*Root (g)*	*Shoot / Root*
Creosote soil (h)[1]	10.4 (1.5)	3.6 (0.9)	2.9 (0.5)
Creosote soil (a)[2]	2.3 (0.2)	1.0 (0.4)	2.3 (0.8)
Mixed soil (h)[1]	9.7 (3.9)	3.1 (0.5)	3.1 (1.2)
Mixed soil (a)[2]	2.3 (0.5)	0.8 (0.2)	2.8 (0.5)
Control (h)[3]	11.9 (1.5)	2.3 (0.5)	5.2 (1.0)
Control (a)[4]	6.1 (0.1)	1.2 (0.5)	5.1 (2.5)

[1)]Means of four healthy plants, [2)]Means of eight affected plants, [3)]Means of ten healthy plants, [4)]Means of two affected plants

The ratio between the shoot and root biomass is 2:1 – 3:1 for willow species (Granhall, 2005 pers.com.) and is to be considered as normal for both healthy and affected plants in both the creosote soil and the mixed soil (Table 4). The ratio in the control soil differed from the contaminated soils with a much higher value of 5.2. In an earlier experiment carried out by Hultgren (2004) the same willow clone showed a shoot-root ratio of 0.5 in a nutrient poor low PAH contaminated soil and a quotient of 0.75 in the control soil. Mastera (2004) measured a ratio of approximately 6:1 in an experiment with a nutrient rich soil spiked with PAHs and the higher ratio in the control soil of this experiment may thus indicate a nutrient rich control soil. Compared with the results of Hultgren (2004) the willow roots in the present trial can be considered as very active, since the root development was limited but plant enhanced PAH degradation was quite impressive. However, the results could also reflect the difficulties of collecting root tissues which was done in different ways in the different experiments. Except for the differences in the shoot-root ratio shoot and root development per se did not differ significantly between controls and healthy plants in the different soils.

Air dried plant material was stored after harvest in unsealed paper or plastics bags in a dry place at room temperature for possible future analyses of conceivable uptake and translocation of the PAHs by the plant.

Chlorophyll content

The color intensity of the willow leaves at 700 nm was measured by a chlorophyll meter (Rexolin Tracer). These data are related to a certain chlorophyll content but since mainly comparisons between the different soil treatments were of interest this transformation was not considered necessary. These measurements were done only on the healthy plants, since the other plants, especially in the creosote soil, were badly affected and data could not be accurately recorded. No obvious differences were found between the bacterial treatments and all plants within the same soil treatment and date were thereby combined irrespective of bacterial treatment. A chlorophyll intensity decrease could be found between plants in the control soil and the contaminated soils, but no difference was found between plants in the mixed soil and the undiluted creosote soil (see Figure 3). Intensity measurements were also made at harvesting time in the pilot experiment conducted during the summer of 2004 and are also shown in Figure (3). Similar values were found for the creosote soil, but in this case the mixed soil differed from the creosote soil but not from the control soil. The conclusion is that the creosote content in the soil seemed to

affect the chlorophyll content of the plant leaves, but concerning the main trial the bacterial infection also has to be taken into account. It can not be excluded that a minor bacterial infection also in the "healthy" plants could explain the overall lower chlorophyll content in the main trial as compared to the pilot trial. In addition to the chlorophyll changes in the pilot experiment other visual signs of contamination effects were observed as well. As mentioned before the biomass production corresponded to the contamination degree of the soil, but also other symptoms as deformed leaves and growth of side shoots could be observed mainly in the undiluted creosote soil.

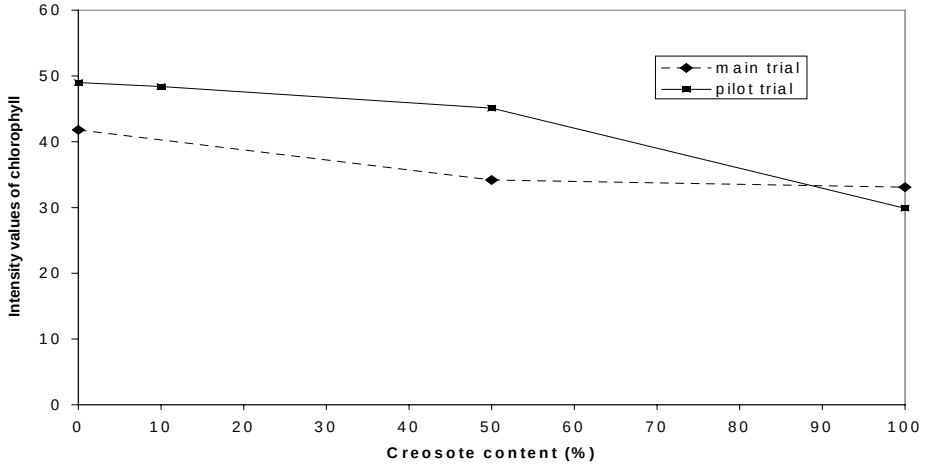


Figure 3. Intensity values of chlorophyll (relative units) for the main trial (broken line) and the pilot trial (solid line) in the soils with different levels of creosote contamination. Means of the healthy plants in the main trial, for four plants in soils with 50- and 100% of creosote content and ten plants in the control soil. Means of two plants in the pilot experiment for all soil dilutions. All together ten measurements independent of the number of plants for each value presented.

Analysis of PAHs

All data are means of the analysed replicates and reported deviations are in most cases based on 95% confidence limits. The method used for the analyses is to quantify the chosen substances, and mainly anthracene, fluoranthene, pyrene and benzo[a]pyrene will be considered. The intention was, as written in the material and methods section, also to follow phenanthrene but this was shown to be too difficult. The very broad ranges in the concentrations of the different PAHs in the soil were not very convenient to handle. Even after dilution the concentrations of some PAHs were too high and this was the reason for sullying the column in the gas chromatograph. The contamination of the column often caused non-reliable peaks in the chromatogram especially for phenanthrene, which was present at relatively low concentrations. The samples were analysed repeatedly several times but this problem remained and when comparing the initial values from the different analytical runs it was found that sometimes these values differed significantly and complicated corrections (see later) had to be done. However, the differences between treatments (for example soil with or without plant) were considered as more important than the absolute concentrations of the individual samples. The results will be presented either as concentrations or as relative values of the compared treatments and when results are presented as concentration values these should not be considered as absolutely

accurate, but rather as reference values. Some samples have been excluded in the further treatment of data due to other experimental errors.

Initial values of the PAH concentration in the soil

As expected the initial values were very high, in particular the fluoranthene and pyrene concentrations (Table 5). This highly aged soil had been subject to various environmental degradations processes, which had changed the contaminant profile and it could be assumed that at least the bioavailable fractions of the more easily degradable two- and three-ring PAHs were degraded to a high extent. Fresh creosote contaminated soil generally contains higher relative amounts of PAHs with three or less aromatic rings (as cited by Lundstedt, 2003).

Comparisons of the initial soil values obtained in this experiment with the results of an earlier study (1992) at the site, carried out by VBB Viak and commissioned by the Swedish railway administration (Banverket) (SwecoVBB Viak, 1995), are given in Table 6.

Table 5. Initial PAHs concentrations of the creosote soil and the mixed soil. All values are presented in ppm and standard deviations are given in parentheses.

Compound (PAH)	*Anthracene*	*Fluoranthene*	*Pyrene*	*Benzo[a]pyrene*
Creosote soil	259 (39)	1014 (107)	1247 (135)	309 (67)
Mixed soil	94 (2)	434 (10)	531 (15)	190 (5)

The standard samples and the samples for determining initial values were analysed several times during the analysis sequence. The standard samples had a larger variation, when analysing the same vial during the runs including the creosote soil compared with those including the mixed soil (data not shown). This indicates that the concentration of the contaminants affected the analytical results to a high extent. Corrections had to be done for the drift of the analytic results caused by the high concentrations in the samples, resulting in sullying the gas cromathographical column.

Table 6. Comparison of initial values from different samplings at the same spot. Sampling 1) VBB VIAK, 1992; Sampling 2) this report.

Compound	*Sampling1* (ppm)	*Sampling 2* (ppm)
Phenanthrene	1500	(52)[2]
Anthracene	280	259
Fluoranthene	960	1014
Pyrene	780	1247
Benzo[a]pyrene	nd[1]	309

[1)] Not determined

[2)] Same replicates, but from an earlier analysis.

The comparisons between the different samplings is done for the same sampling spot but at different times. Sampling 2 was made in 2004 and soil from sampling 1 was collected in 1992. Sampling 1 was thus made only a few years after the wood impregnation site was in use. The concentrations of the more insoluble compounds, such as fluoranthene

and pyrene, have not changed noticeably during this twelve-year period, but phenanthrene concentrations have dropped dramatically. Fresh creosote contaminated soil contains large relative amounts of lighter compounds such as phenanthrene, but these compounds are now supposed to be biodegraded to a high extent, so these results are not surprising.

Both in this experiment and a pilot experiment performed by Pizzul (pers.com.) the creosote soil was diluted at the ratio 1:1 with the same control soil, from Ulleråker. After soil dilution the concentrations of the compounds are not expected to be exactly half of the undiluted creosote soil, but the ratio should be the same for all compounds if the actual soil mixing affects the compounds in the same way. By comparing the relationships between the compounds in the mixed soil with those of the undiluted creosote soil in the two experiments the concentrations were found to be approximately halved for phenanthrene, fluoranthene and pyrene (see Table 7). Anthracene, in Exp. 1, had a slightly lower ratio and for benzo[a]pyrene the ratio was about 0.6 in both cases. For benzo[a]pyrene this indicates that a higher proportion was extractable after mixing the soil. These results are unexpected and no likely reason for this is found. Solubility mechanisms resulting from microbial activity could be neglected due to the short time. Chemical desorption processes in soil have been found to be biphasic with a rapid and a slow fraction desorption rate following first order kinetics (Correlissen, 1998). The results may thus be explained by thermodynamic desorption reactions, but this is not evident because our creosote soil is an aged soil and compounds desorb slowly in such soils (Correlissen *et al.*, 1998). This interpretation is also contradicted by the results found for the other more soluble PAHs.

Table 7. Dilution rate due to soil mixing of the different compounds in the trials; The concentrations of the mixed soil are divided with the concentration of the creosote soil. Exp. 1) this experiment; Exp. 2) Pizzul, 2005 pers.com.

	Phenanthrene	*Anthracene*	*Fluoranthene*	*Pyrene*	*Benzo[a]pyrene*
Exp. 1	(0.46)[1]	0.36	0.43	0.43	0.61
Exp. 2	0.53	0.45	0.47	0.48	0.60

[1)] Same replicates, but from an earlier analysis.

Sampling after week 4 and 8

Sampling, extraction and analyses of soil after four or eight weeks were made as planned, but since the results showed a very unrealistic and confusing pattern these results will not be presented. When all sources of errors were investigated it was concluded that this was likely a result due to insufficient mixing of the soil before starting the trial.

Final sampling, extraction and analysis

Because of the bacterial infection the final sampling and the harvesting of the plants was made two weeks earlier than planned, i.e. at week 10.

Bacterial inoculations

The results indicate that the introduction of PAH degrading bacteria was not successful. No difference was found between the bacterial treatments in pots without plants (n=4 for each treatment) after ten weeks (data not shown). Such results are not uncommon. Many

laboratory experiments have shown that inoculation was unnecessary. The soil aeration and other factors affecting the degradation of the pollutants are in many cases of more importance (Alexander, 1999). Still the survival and degradation activity of the inoculated *Rhodococcus wratislaviensis* and J04 in this study are not known. At the end of the experiment none of the bacterial strains were isolated and identified, neither from the unplanted soil or from the rhizosphere soil. From these results it can neither be confirmed nor excluded that these strains are suitable for bioremediation of PAHs.

Treatments without plants – irrespective of bacterial treatment

The analysed compounds in pots without plants after ten weeks of treatment are shown in Table 8. In the unplanted soil the concentrations of fluoranthene and pyrene had decreased in the creosote soil by 35 and 19 % respectively but the disappearance of anthracene was not statistically significant and benzo[a]pyrene was, as expected, not different from the initial value.

Table 8. Comparison between initial concentrations and after ten weeks of treatment without plants. All values are presented in ppm and 95% confidence limits are given in parentheses.

Compound	*Creosote soil*		*Mixed soil*	
	Initial[1]	*After 10 weeks*[2]	*Initial*[1]	*After 10 weeks*[3]
Anthracene	275 (39)	222 (30)	95 (4)	95 (11)
Fluoranthene	1014 (107)	656[*] (68)	434 (10)	733[*] (64)
Pyrene	1247 (135)	1011[*] (89)	525 (15)	625[*] (60)
Benzo[a]pyrene	309 (67)	350 (37)	190 (5)	195 (13)

[1] (n =4), [2] (n=10), [3] (n=10), [*] significant difference from initial concentrations ($P< 0.05$).

In the mixed soil without plants anthracene and benzo[a]pyrene were not different from the initial values, but compared to the initial soil the concentration of fluoranthene had increased with almost 70% and pyrene with slightly less than 20 %. Since no PAHs were added, these results have to be explained by an increased solubility during the experiment or that a larger fraction of the contaminants was extracted. The organic content of the control soil was 1.8 % (Stenström *et al.*, 2001) but was not determined for the other soils. However, it is assumed that the control soil had a lower organic content and the mixed soil would thus have a lower organic content compared to the undiluted creosote soil. This may have caused a higher PAH desorption in the mixed soil and resulted in an increased extractable fraction of these compounds, but would not explain the higher concentrations of fluoranthene and pyrene. The PAH solubility may also be enhanced in the mixed soil, due to a slightly higher water content, but this will hardly explain such large concentration increases. However, metabolite formation (anthraquinone) indicates that both degradation and increased solubility occurred at least concerning anthracene (see later).

Plant effect on PAH degradation

The concentrations of all four compounds in the planted creosote soil had dropped dramatically after ten weeks, including benzo[a]pyrene which showed a reduction from 309 ppm to 176 ppm in the presence of *Salix viminalis.* The plant effect was obvious in the mixed soil as well, especially concerning fluoranthene and pyrene. The initial values

of the analyzed compounds were compared with the results after ten weeks in unplanted and planted soil in the creosote soil (Figure 4) and in the mixed soil (Figure 5).

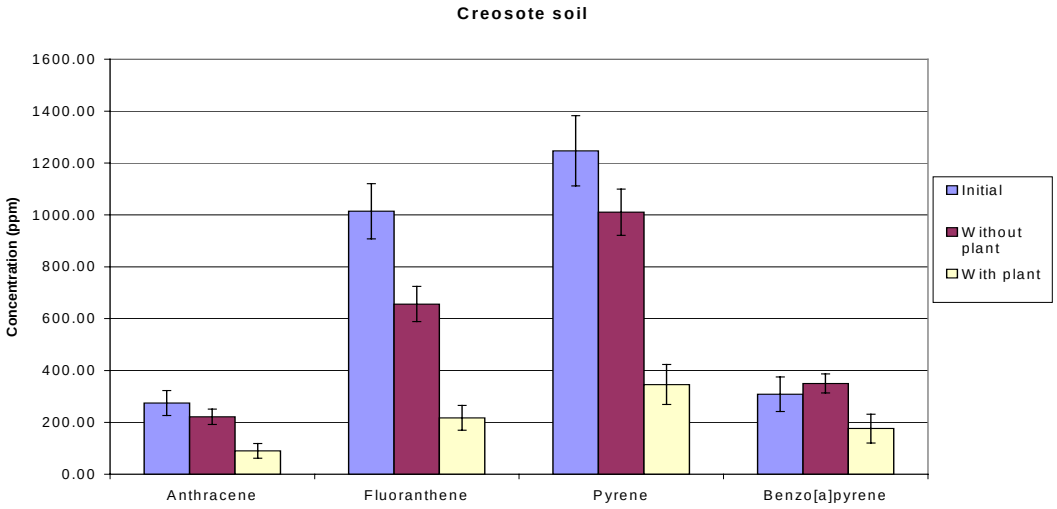


Figure 4. Comparison between the initial values and after ten weeks of treatment with or without plants in the creosote soil. Initial values (n = 4), treatment without plants (n = 10) and treatment with healthy plants (n = 4). Bars show 95 % confidence limits.

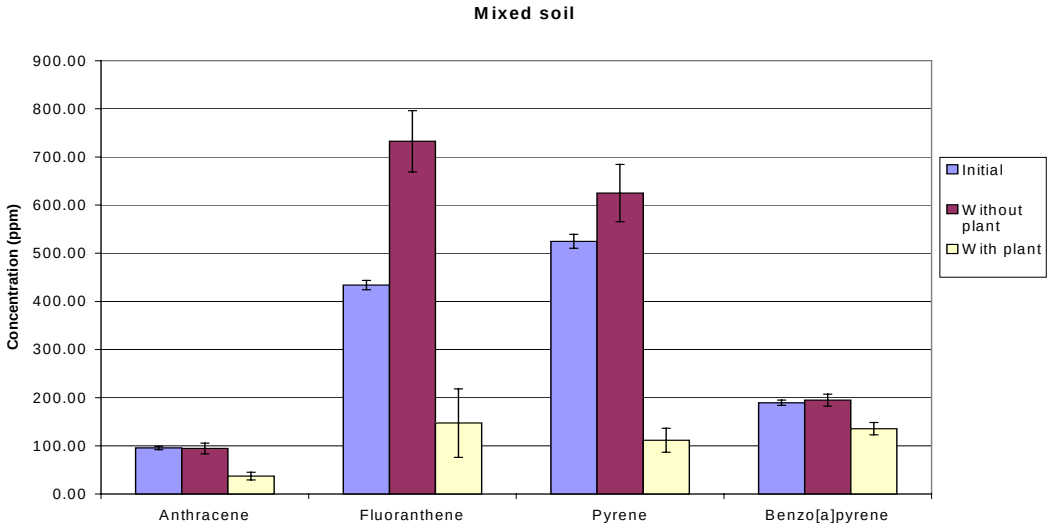


Figure 5. Comparison between the initial values and after ten weeks of treatment with or without plants in the mixed soil. Initial values (n = 4), treatment without plants (n = 11) and treatment with healthy plants (n = 4). Bars show 95 % confidence limits.

The fact that the plants enhanced PAH degradation is interesting and promising. Benzo[a]pyrene, which is one of the most recalcitrant PAH compounds was reduded, by 43 % in the creosote soil and by nearly 30 % in the mixed soil. The other PAH compounds were reduced even more with 67 %, 79% and 77% for anthracene, fluoranthene and pyrene respectively in the creosote soil and with 61 %, 66% and 79% in

the mixed soil. The results are impressive especially considering the very low root production and that root development did not occur throughout the entire pots.

The root production may not have been very dense but the results indicate that the roots present had been very active. In this experiment a correlation between the biomass production and the degradation rate was established, but it is not known to what extent other plant properties are involved in the biodegradation of PAHs. Newman & Reynolds (2004) refer to a trial carried out with poplar and PAH degradation where the authors found that the poplar cuttings grown in a moderate PAH contaminated sand-nutrient solution had similar above ground biomass, growth and leaf water content as the control, but it was found that transpiration, nutrient uptake and root biomass were reduced. If plants are stressed, like in this trial due to the bacterial infection and the contaminated soil, this will probably affect the PAH degradation in different ways. For example there might be changes in the plant derived production of biosurfactants. Also plant peroxidases may increase in stressed plants (Ollerstam, 2002). A low root production may thus not be the same as a low rhizosphere effect influencing the PAH degradation.

The fate of the disappeared PAHs is unknown, but the possibilities are numerous. Repeated analytic runs gave different values, but did not change the pattern and the plant effect was clear. The plants may have contributed to the enhanced PAH degradation through a stimulation of the bacterial community by releasing easily available carbon- and nutrient compounds (biostimulation). The plants may also favour the soil conditions for the microorganisms through increased oxygen supply or physical changes (bioventing) as a result of the root development.

The soil was a highly aged creosote soil where degradations processes have been going on for many years and changed the composition of the contaminant profile in favour of the more recalcitrant compounds. Since high molecular weight PAHs mainly are degraded through cometabolic processes, degradation of these compounds may have been inhibited with declining concentrations of lighter PAHs. This hypothesis was presented by Rentz *et al.* (2005), who also suggested that high molecular weight PAHs may not be sufficiently remediated through rhizosphere processes and claimed that an increased number of bacteria in the root zone is not enough if the bioavailable selective pressure for PAH degraders is limited.

The introduction of plants may, on the other hand, increase the solubility of not bioavailable PAHs including those with a low molecular weight through the release of biosurfactants. This will increase the degradation potential of the more recalcitrant PAHs since desorbed low molecular weight PAHs will be used as substrate by bacteria and heavier PAHs will be degraded cometabolically at the same time. The PAHs may have been adsorbed to the roots or absorbed into the root tissues but even if this is the case it will probably contribute very little to the disappearance of PAHs (Miya & Firestone, 2001).

Enhanced PAH degradation in the presence of *Salix viminalis* is in keeping with earlier investigations. Mastera (2004) found an increased disappearance of phenanthrene,

anthracene, pyrene and benzo[a]pyrene in a spiked soil in the presence of the same willow clone (clone 183) but not when using the clone Gudrun and Hultgren (2004) established an enhanced degradation rate of phenanthrene and pyrene in treatments with clone 183 of *Salix viminalis* in a low creosote contaminated soil.

The aged creosote soil has been subject to various environmental degradation processes which have, as mentioned before, changed the contaminant profile in favour of more recalcitrant compounds. The persistence of mainly insoluble compounds in the soil was expected. Fluoranthene and pyrene are both built up with four benzene rings and even though the water solubility of fluoranthene is higher than the solubility of pyrene (0.260 mg/ l compared with 0.140 mg /l) the fate of these compounds is often considered as comparable. The ratio between fluoranthene and pyrene was approximately 0.8 in the initial soil (see Table 9 and 10 below), both for the undiluted and diluted case, but the relation between these two compounds changed during the experiment and also between the different soils. In the creosote soil the degradation of fluoranthene seemed to be favoured during the experiment and the ratio decreased to approximately 0.6 (Table 9). This may be explained by the higher water solubility of fluoranthene. No difference was found in this ratio between the treatments with-, or without plants or between the planted bulk soil and the rhizosphere soil. In the mixed soil, however, the pattern was reversed and the ratio had increased by the end of the experiment showing a decreased degradation rate of fluoranthene compared to pyrene, which is not different per se from the dissipation rate of the creosote soil, in treatments both with and without plants (Table 10). In the mixed soil the dissipation rate of fluoranthene was also restricted in the rhizosphere but to a lower extent and also here the pattern was reversed to the creosote soil. Explanations for this are only speculative. The dilution changed the water content of the soil which should be in favour of fluoranthene solubility. The mixing may also have changed the composition of the microbial community and in some way stimulated pyrene degraders. Since this seemed to be a result of the mixing and not due to the introduction of the plant, it is likely that it can be referred to the chemical or physical properties of the soil itself like changes in soil pH or organic content. When diluting the creosote soil the pH would increase but the organic content would decrease. If and to what extent these changes have affected the degradation of fluoranthene is not known. However, Pizzul (unpubl.) found that the dissipations of fluoranthene and pyrene were interrelated in a similar way in both the mixed soil and the creosote soil.

Table 9. Relations between fluoranthene and pyrene in the creosote soil. The values of fluoranthene and pyrene are presented in ppm.

	Initial	*Without plant*	*With healthy plant*	*Rhizosphere*
Fluoranthene	1014	656	218	382
Pyrene	1247	1011	346	652
Flu/Pyr	0.81	0.65	0.63	0.58

Table 10. Relations between fluoranthene and pyrene in the mixed soil. The values of fluoranthene and pyrene are presented in ppm.

	Initial	*Without plant*	*With healthy plant*	*Rhizosphere*
Fluoranthene	434	733	147	247
Pyrene	531	625	112	256
Flu/Pyr	0.82	1.38	1.32	0.97

PAH concentrations in the rhizosphere

The concentrations in the rhizosphere soils were found to be greater for all studied PAHs than in the bulk soils, but with a high variation between the replicates (Table 11). This could be explained mostly by the movement of compounds from the surrounding soil into the rhizosphere, but partly also by increased solubility. When comparing results of healthy and affected plants the concentrations in the creosote soil had increased even more in pots with affected plant. This was valid for all studied PAHs. Except for pyrene , a similar pattern was found in the mixed soil. The higher concentration increases in pots with affected plant are likely to be a result of lower degradation rates of the compounds in these pots, compared with pots containing healthy plants.

Table. 11 Means of the increased concentrations (%) in the rhizosphere soil compared with the bulk soil. Values in parentheses are 95% confidence limits. (h): healthy plants; (a): affected plants.

	Anthracene	*Fluoranthene*	*Pyrene*	*Benzo[a]pyrene*
Creosote soil (h)[1]	87 % (54)	84 % (55)	97 % (55)	62 % (31)
Creosote soil (a)[2]	115 % (54)	143 % (25)	165 % (27)	86 % (41)
Mixed soil (h)[1]	64 % (26)	83 % (39)	125 % (71)	37 % (15)
Mixed soil (a)[3]	96 % (41)	121 % (50)	80 % (64)	121 % (51)

[1)] Means of four plants; [2)] Means of seven plants; [3)] Means of eight plants

Increased PAH concentrations in the rhizosphere have been commonly reported. Liste and Alexander (2000b) found that the rhizosphere soil of soybean contained significantly more phenanthrene than unplanted soil after 14 days. The concentration of phenanthrene was 34% higher compared to the day zero level and almost four times higher than the unplanted soil after 14 days. The accumulation of phenanthrene in the rhizosphere declined and after 42 days the concentration of phenanthrene in the rhizosphere was similar to that in the unplanted soil. The same pattern occurred with the concentration of pyrene in the alfalfa rhizosphere after 14 days. After 79 days, however, the pyrene concentrations in the rhizosphere and the unplanted soil were not significantly different.

Increased concentrations in the rhizosphere may be explained by the plant's influence on the soil water content and water movement in the soil. Transportation of mobile, more soluble, PAHs may be enhanced by the water movement to the root zone. This explination is partly contradicted by our data, showing increased concentrations also of compounds with a very low water solubility, such as benzo[a]pyrene. Another explanation of the increased concentrations in the rhizosphere could be an increased solubility of the compounds due to release of plant produced surfactants. If on one hand, the PAH solubility is enhanced by biosurfactants produced by the stressed plant, but on the other the degradation rate is negatively affected due to plant stress, increased concentrations may be found in the plant rhizosphere. This would particularly be the case

for plants affected by the bacterial infection and its negative impact on the microbial activity.

Also in the experiment of Mastera (2004), benzo[a]pyrene tended to be more concentrated in the rhizosphere than in the bulk soil. In this experiment increased concentrations of benzo[a]pyrene were also found in a treatment with addition of a surfactant (Triton) indicating an enhanced solubility at the same time as the degradation was very restricted.

Comparison between the soils
The justification for the soil dilution was mainly to reduce the toxicity of the soil in order to:

i) Investigate whether Salix viminalis was affected by the PAH concentration and how this could affect the PAH degradation indirectly and

ii) Test if the dilution itself could enhance the PAH degradation by changing the properties of the soil

In treatments without plants, as mentioned above, no positive effect on dissipation was found after soil dilution (Figure 7). The concentrations of foremost fluoranthene and pyrene had rather increased. Increased concentration of anthraquinone but no decreased concentration of anthracene support the hypothesis that increased solubility could be true for other PAH compounds as well. Reasons for such results are poorly understood and the importance of the soil itself is not demonstrated. When mixing the soils the water content increased compared to the undiluted creosote soil. This may have increased the solubility of some compounds and also enhanced the microbial degradation since the biodegradation should have mainly occurred in the water phase (Johnsen *et al*., 2005). Pizzul (unpubl.) on the other hand found the dissipation of PAHs in mixed soil to be the same as in undiluted creosote soil. Her experiment was carried out using soils, which were comparable to those used in the present study.

In treatments with plants an enhanced PAH degradation was found in both the creosote soil and the mixed soil, but the dissipation of the compounds was not enhanced by the soil dilution. The opposite was in fact found for benzo[a]pyrene, which had a lower degradation rate in the mixed soil compared to the creosote soil (see Figure 6 and 7).

The assumption that the soil dilution would enhance the PAH degradation is not confirmed by these results or those reported by Pizzul (unpubl.).

Metabolite formation
The PAH metabolite anthraquinone was followed throughout the experiment as a reference metabolite as the relation between anthraquinone and anthracene has been studied earlier, among others by L. Pizzul at our department, and shown to be interrelated in the sense that the degradation of anthracene and the formation of anthraquinone are positively correlated. Antraquinone formation from anthracene is mainly a result of

fungal degradation, where extracellular peroxidases of fungi are responsible for the initial oxidation of the PAH (Kästner *et al.*, 1999; Lundstedt, 2003). However, also chemical reactions yield antraquinone from anthracene (Mallakin *et al.,* 2000; Brack *et al.,* 2003). Anthracene degradation pathways may also result in other degradation metabolites such as benzoic acids, benzaldehydes and phenols (Mallakin *et al*., 2000).

In this study no clear relationship was found. There was often an opposite trend, i.e. the concentration of anthraquinone decreased concomitantly with a decrease in anthracene (Table 12). It is important, however, to consider that Pizzul used a spiked soil adding fresh PAHs and in my case an aged contaminated soil was used, where anthraquinone was present in the initial soil. It is therefore assumed that both degradation and formation of anthraquinone are taking place during the experiment and that the net concentrations may not be different from the disappearance of anthracene. When comparing the dissipitation rates (Table 13) it was found that the quotient of anthracene /anthraquinone in the treatment without plants was not different from the initial values either in the creosote soil or in the mixed soil, even though the quotient itself differed. In the treatment with plants, however, this quotient had changed in both the creosote soil and the mixed soil indicating that the net concentrations of anthraquinone were lowered compared to the degradation of anthracene.

No difference was found in the concentrations of anthracene in the mixed soil between the initial values and the values after ten weeks of treatment without plant. However, increased concentrations of antraquinone were found compared to the initial values. Since no formation of anthracene could possibly have taken place the increased concentrations of anthraquinone are supposed, at least partly, to originate from anthracene degradation. This result supports the assumption that an increased solubility of several PAHs occurred in the mixed soil.

Table 12. Mean values of the concentration of anthracene and antraquinone in the beginning of the trial and after ten weeks with- or without plant. Values in parenthesis are 95% confidence limits.

	Anthracene		*Anthraquinone*	
	Creosote soil	Mixed soil	Creosote soil	Mixed soil
Initial	275 (48)	95 (4)	241 (45)	123 (5)
Without plant	222 (47)	95 (11)	195 (37)	136 (16)
With plant	91 (29)	37 (8)	120 (39)	89 (9)

Table 13. Comparison of quotients of anthracene and anthraquinone in the creosote soil and the mixed soil.

	Anthracene / Anthraquinone	
	Creosote soil	Mixed soil
Initial	1.14	0.78
Without plant	1.14	0.70
With plant	0.75	0.41

Microbial analyses

It was hypothesised that the increased degradation in the pots with plants could be the result of a stimulation or changes in the microbial community or as a result of plant produced biosurfactants and enzymes.

Description of bacterial population

Soil bacteria were extracted and isolated from the creosote soil and the initial soil was compared with treatments with and without plants. In treatments with plants bacteria were extracted from the rhizosphere soil and both soil from pots with affected plants and healthy plants was included. The number of extracted and cultivable bacterial colony forming units (CFU) per gram of soil (dw) are presented in Table 14. All results are based on two replicates from the same soil sample and the differences are thus only to be considered as trends. . The number of bacteria was higher after ten weeks of treatment in the pots without plants compared to the initial creosote soil. The bacterial populations increased even more in all treatments with plants compared to the initial values. Healthy plants were found to have a much higher number of bacterial colonies compared to samples from pots without plants, but in pots with affected plants this relation was not significant. The higher number of colonies for J04 (h) can either represent the single soil sample or reflect the treatment in general.

Table 14. Bacterial colonies per gram dw. All values should be multiplied with 10^6. (h); healthy plants, (a); affected plants. Values in parentheses are standard deviations from mean values of two analyses.

	Initial	*Without plants*	*Control*[1]		*R.wratislaviensis*		J04	
			(h)	(a)	(h)	(a)	(h)	(a)
CFU/ g dw	0.54 (0.035)	1.08 (0.028)	1.59 (0.035)	0.92 (0.064)	1.59 (0.042)	0.93 (0.25)	6.59 (0.21)	0.90 (0.007)

[1)] No bacterial inoculation

The conclusion of the bacterial population counts is that the bacterial numbers seem to increase in all the treatments compared to the initial soil and that this increase is enhanced by the presence of healthy willow plants. The tendency was confirmed when combining the bacteria treatments to get more replicates and despite the extreme values of J04 (h) a significantly higher cultivable number of bacteria colonies per gram of soil was extracted from pots with healthy plants compared to pots with affected plants (see Figure 6).

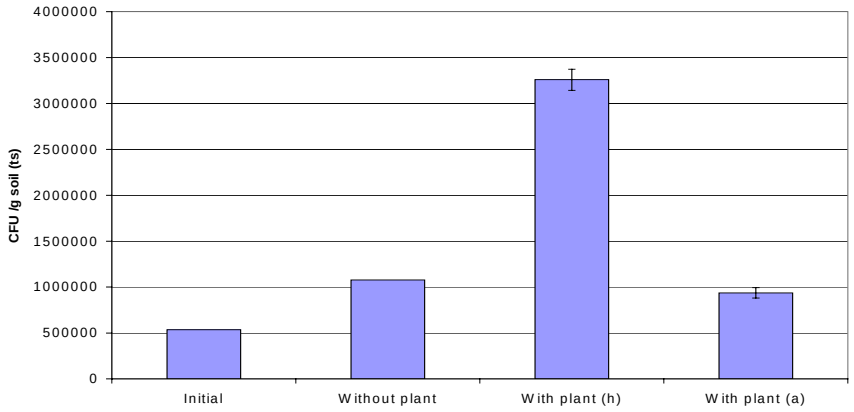


Figure 6. Bacteria colony forming units in the creosote soil comparing the initial soil with treatments after ten weeks, both with and without plants. The bacterial treatments are combined and results of healthy and affected plants are shown separately. (h); healthy plants, (a); affected plants. Bars show 95 % confidence limits.

The enhanced degradation in the planted soil could thus be explained by an increased microbial activity in the pots with plants and possibly a greater release of biosurfactants and higher numbers of bacteria able to degrade the PAHs. These assumptions are supported by the results of Hultgren (2004) and Mastera (2004) where not only a higher number of bacterial populations was found in treatments with plants, but also an increased number of active microbes.

Description of bacterial groups

The most common, or in other ways interesting, cultivable bacteria strains from the initial creosote soil and the creosote soil after treatment with or without plant were isolated and grouped based on tests of gram- fluorescence- and oxidase reactions and these groups are shown in Table 15. These isolated strains were stored at -70°C for further tests regarding their ability to degrade PAHs.

Table 15. Groups of isolated soil bacteria based on the functional tests of gram-, fluorescence and oxidase reactions (Hultgren, 2004).

Group Number	*Gram reaction*	*Fluorescence reaction*	*Oxidase reaction*	*Bacterial examples*	*No. of isolates*
1	Positive	Positive	Positive	*Bacillus*	-
2	Positive	Negative	Positive	*Bacillus*	4
3	Positive	Positive	Weak positive	*Bacillus*	-
4	Positive	Negative	Weak positive	*Bacillus*	-
5	Positive	Positive	Negative		-
6	Positive	Negative	Negative	*Actinomycetes, Arthobacter, Coynebacterium*	4
7	Negative	Positive	Positive	*P. fluorescens*	2
8	Negative	Negative	Positive	*Sphingomonas*, *P.*spp	2
9	Negative	Positive	Weak positive	*P.viridiflava*	-
10	Negative	Negative	Weak positive	*Xanthomonas*	3
11	Negative	Positive	Negative	*P. syringae*	-
12	Negative	Negative	Negative	*Enterobacteriaceae*	1

No differences in occurrence could be found between the treatments considering the dominant bacterial groups, but the order of dominance differed (Table 16). In the initial soil group number 8 was dominating and this was also the case for the soil with non-inoculated healthy plants. For the other groups number 2 was dominating. In general the most frequent bacterial species belonged to group numbers 2, 8 and 10 and in all treatments bacterial isolates from groups number 2 and 10 are represented among the three most abundant ones. Examples of bacterial species belonging to these groups are *Bacillus* and *Xanthomonas* respectively. It is not surprising to find these groups as the dominating ones since in fact both *Bacillus* and *Xanthomonas* are well documented as PAH degraders (Mueller *et al.*, 1996; Ahn *et al*, 1999). Group number 6, however, is rarely represented among the dominating groups and its distribution is not supported by earlier results (Hultgren, 2004; Mastera, 2004) where bacterial strains belonging to this group were dominating both in planted and unplanted PAH contaminated soil.

Table 16. Dominating bacterial groups among the treatments referring to Table 15 above. No quantifications are made.

Order of dominance	*Initial*	*Without plant*	*Control*		*Rhodococcus*		*J04*	
			Healthy	Affected	Healthy	Affected	Healthy	Affected
I	8	2	8	2	2	2	2	10
II	10	2	2	10	8	10	10	6
III	2	10	10	2	2	10	8	

Sources of errors

Insufficient mixing of the soil before starting the experiment may have caused some of the problems. The uneven mixing of the soil is probably one reason why the results from the sampling at week four and eight did not agree with the initial values and the final sampling. When preparing the diluted soil the soil was mixed more carefully and this might be the reason for smaller differences in the initial values among the replicates in the mixed soil compared to the initial soil of the undiluted creosote soil.

Since the samples were diluted before analysis a small error in the analyses was multiplied when recalculations were made.

The high concentration of the contaminants in the analysed samples sullied the column of the gas chromatograph and this affected the accuracy of the results.

At harvest, the plants were removed from the soil and gently shaken to remove soil loosely attached to the roots. The rhizosphere soil samples, may contain relatively higher amounts of roots than the bulk soil of planted pots and thereby higher PAH concentrations since PAHs may adsorb to, or be absorbed by the roots.

CONCLUSIONS and FUTURE PERSPECTIVES

Salix viminalis accelerated the dissipation of all the analysed PAHs: anthracene, fluoranthene, pyrene and benzo[a]pyrene in both the undiluted creosote soil and the mixed soil, despite the fact that the bacterial infection had suppressed root and shoot development. The reduction was impressive for all compounds, but the disappearance of benzo[a]pyrene was of particular interest due to the recalcitrant properties of this high molecular weight PAH.

The highly contaminated soil caused several analytical problems. These problems may be partly solved by further dilution of the samples, but the broad concentration spectra of the different compounds will still be a problem. It is of importance to keep the analytic accuracy and at the same time be able to determine several compounds. Several institutes, departments and companies are working with these kinds of highly contaminated soils, and for the future research here at the Department of microbiology at SLU it is of concern to work with methods giving trustable and reproducible results.

The absolute initial values of PAHs of the aged creosote soil are not fully known since they depend on the efficiency of the extraction method. The efficiency of the extraction method has been evaluated in a spiked soil (Pizzul, pers. com.), but is not necessarily comparable with that of an aged creosote soil. For future work with such a highly contaminated creosote soil also the method used for extraction has to be further evaluated.

The found increased PAH concentrations in the rhizosphere are supported by several reports suggesting an effect of the water flow towards the root zone. This hypothesis is contradicted by increased concentrations including compounds with a very low water

solubility, such as benzo[a]pyrene, and further investigations are necessary to evaluate this phenomenon. The increased rhizosphere concentrations may also be related to plant biosurfactant release, but the mechanisms for such a release are poorly understood.

The use of *Salix viminalis* in phytoremediation applications is promising, but it will be necessary to evaluate the potential of other clones to reduce soil toxicity. The clone used in this experiment has affected PAH degradation, indirectly or directly, but it is also confirmed that this clone is susceptible to bacterial infections. In general creosote contaminated soils contains high levels of trace metals and clones both able to reduce the PAHs levels in the soil and being successful in trace metal uptake should be desirable. The impact of willow trees for remediation of organic pollutants and plant uptake of trace metals have been studied with positive results, but the effect of willow trees on the fate of PAHs in soil is less well documented and much has yet to be learned about the microbiology of the willow rhizosphere and its relation to PAH removal. PAH uptake by plants has been documented for other species, such as maize and wheat, but this phenomenon is of high interest to evaluate also concerning willow.

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APPENDIX

The figure shows a map of the sampling site at Krylbo. The sampling spot is indicated with L1.

APPENDIX

Table describing the gram-, fluorescence- and oxidase reaction of the bacteria isolates from the willow cuttings. Bact A: *Rhodococcus wratislaviensis*, Bact B: J04.

Group number[1]	*Gram reaction*	*Fluorescence reaction*	*Oxidase reaction*	*Isolate number*	*Treatment*
2	Positive	Negative	Positive	K18	Mixed soil + Bact B
7	Negative	Positive	Positive	K1	Creosote soil
7	Negative	Positive	Positive	K3	Creosote soil
7	Negative	Positive	Positive	K4	Creosote soil + Bact A
7	Negative	Positive	Positive	K10	Mixed soil
7	Negative	Positive	Positive	K15	Mixed soil + Bact A
7	Negative	Positive	Positive	K33	Creosote soil + Bact A
7	Negative	Positive	Positive	K36	Creosote soil + Bact B
7	Negative	Positive	Positive	K37	Control soil
7	Negative	Positive	Positive	K38	Control soil
7	Negative	Positive	Positive	K41	Control soil+ Bact B
8	Negative	Negative	Positive	K7	Creosote soil + Bact B
8	Negative	Negative	Positive	K9	Creosote soil + Bact B
8	Negative	Negative	Positive	K12	Mixed soil
8	Negative	Negative	Positive	K14	Mixed soil + Bact A
8	Negative	Negative	Positive	K16	Mixed soil + Bact B
8	Negative	Negative	Positive	K28	Creosote soil
8	Negative	Negative	Positive	K29	Creosote soil
8	Negative	Negative	Positive	K30	Creosote soil
8	Negative	Negative	Positive	K32	Creosote soil + Bact A
8	Negative	Negative	Positive	K39	Control soil + Bact A
8	Negative	Negative	Positive	K40	Control soil + Bact A
8	Negative	Negative	Positive	K42	Control soil+ Bact B
10	Negative	Negative	Weak pos/neg	K11	Mixed soil
10	Negative	Negative	Weak pos/neg	K13	Mixed soil + Bact A
10	Negative	Negative	Weak pos/neg	K17	Mixed soil + Bact B
12	Negative	Negative	Negative	K2	Creosote soil
12	Negative	Negative	Negative	K5	Creosote soil + Bact A
12	Negative	Negative	Negative	K6	Creosote soil + Bact A
12	Negative	Negative	Negative	K8	Creosote soil + Bact B
12	Negative	Negative	Negative	K31	Creosote soil + Bact A
12	Negative	Negative	Negative	K34	Creosote soil + Bact B
12	Negative	Negative	Negative	K35	Creosote soil + Bact B

1) Groups according to Hultgren, 2004.